Research article

4-(N,N-dipropylamino)benzaldehyde inhibits the oxidation of *all-trans* retinal to *all-trans* retinoic acid by ALDH1A1, but not the differentiation of HL-60 promyelocytic leukemia cells exposed to *all-trans* retinal

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Abstract

Background: The signal transduction pathways mediated by retinoic acid play a critical role in the regulation of cell growth and differentiation during embryogenesis and hematopoiesis as well as in a variety of tumor cell lines in culture. Following the reports that two members of the superfamily of aldehyde dehydrogenase (ALDH) enzymes, ALDH1A1 and ALDH1A2, were capable of catalyzing the oxidation of *all-trans* retinal to *all-trans* retinoic acid with submicromolar K_m values, we initiated an investigation of the ability of 4-(N,N-dipropylamino)benzaldehyde (DPAB) to inhibit the oxidation of retinal by purified mouse and human ALDH1A1.

Results: Our results show that DPAB potently inhibits retinal oxidation, with IC_{50} values of 0.11 and 0.13 µM for purified mouse and human ALDH1A1, respectively. Since the HL-60 human myeloid leukemic cell line has been used extensively to study the retinoic acid induced differentiation of HL-60 cells to granulocytes, and ALDH1A1 activity had previously been reported in HL-60 cells, we investigated the ability of DPAB to block differentiation of HL-60 promyelocytic leukemia cells exposed to retinal in culture. In HL-60 cells coincubated with 1 µM retinal and 50 µM DPAB for 144 hours, cell differentiation was inhibited only 30%. Furthermore, the NAD-dependent oxidation of propanal or retinal was less than 0.05 nmoles NADH formed/min-10^7 cells in spectrophotometric assays using HL-60 cell extracts.

Conclusions: Although ALDH1A1 may be the major catalytic activity for retinal oxidation in some retinoid-dependent mouse and *Xenopus* embryonic tissues and in adult human and mouse hematopoietic stem cells, another catalytic activity appears to synthesize the retinoic acid ligand necessary to stimulate the differentiation of HL-60 cells to end stage granulocytes.
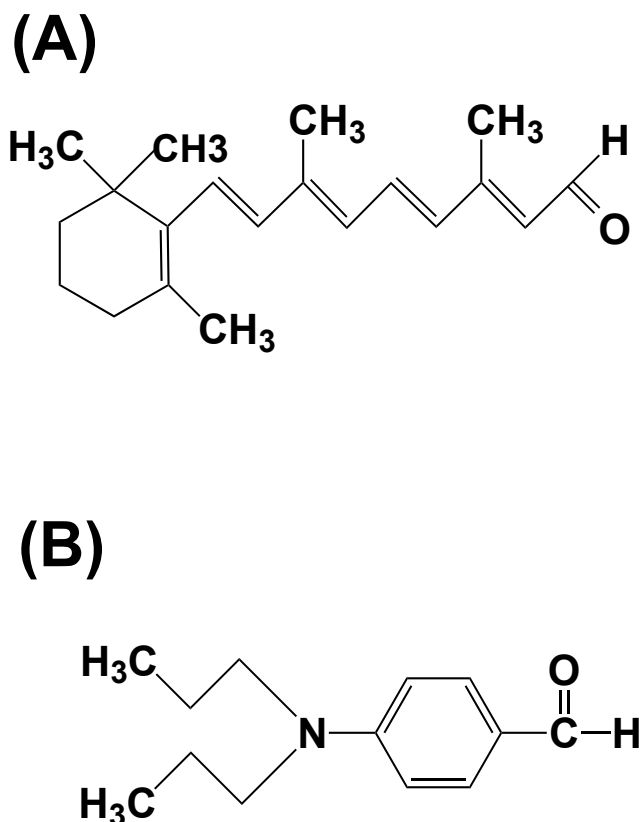
(A)

(B)

Figure 1
Chemical structures for ALDH1A1 substrate and inhibitor. **(A)** substrate: *all-trans* retinal. **(B)** inhibitor: 4-(N,N-dipropylamino)benzaldehyde (DPAB).

Background

The aldehyde dehydrogenase (ALDH) superfamily of enzymes consists of a group of NAD(P)-dependent enzymes which can oxidize a structurally diverse group of endogenous and exogenous aldehyde substrates. Seventeen *ALDH* genes have been identified in the human genome and have been classified into ten different families based on amino acid sequence identities [1,2]. High levels of ALDH1A1 activity can protect human and murine cells from the toxicity of the alkylating agent cyclophosphamide and its oxazaphosphorine analogs. Indeed it was hypothesized that the high expression of ALDH1A1 (formerly known as class 1 ALDH) was responsible for the successful use of cyclophosphamide in tumor cell purging regimens during bone marrow transplantation [3]. The detection of elevated ALDH1A1 activity in the most primitive human hematopoietic progenitor cells [4–6] has provided evidence in support of this hypothesis. However, no physiologic role for this enzyme in the hematopoietic stem cell population has been identified.

Vitamin A (retinol) is the prototype of a class of natural and synthetic chemical compounds named the retinoids. Retinoids are found in a variety of chemical oxidation states, including alcohol, aldehyde, ester, and carboxylic acid functionalities. The acid form, retinoic acid, is a pleiotropic hormone which regulates gene expression in embryonic development, epithelial cell differentiation, hematopoeisis, and tumor cell formation.

Several enzymes, including isoforms of alcohol dehydrogenase, ALDH, and cytochrome P450 (CYP), recently have been shown to be involved in the formation of retinoic acid from retinol and retinal [7,8]. The purification, cloning, and characterization of several ALDH enzymes which efficiently catalyze the oxidation of retinal (Figure 1A) has suggested that regulation of retinoic acid formation could be a key physiologic role for these enzymes. The report of the crystal structure of the homotetrameric ALDH1A1 enzyme from sheep liver revealed a substrate tunnel capable of binding retinal which was absent in ALDH2 and ALDH3A1 enzymes [9]. Likewise, the crystal structure reported for mouse ALDH1A2 revealed a substrate channel which could provide specificity for retinal and exclude short-chain aliphatic aldehydes [10].

The specific role of each enzyme in the regulation of retinoid signalling may depend on the species, cell type, and developmental status of the cell. *In vitro*, purified mouse [11], human [12], and *Xenopus*[13] ALDH1A1 can efficiently oxidize *all-trans* retinal to *all-trans* retinoic acid. *In vivo*, the introduction of ALDH1A1 mRNA into *Xenopus embryos* induces early synthesis of retinoic acid, while ALDH1A1 expression is detected during the tail bud stages of *Xenopus* embryogenesis [14]. ALDH1A2 in the mouse [15] and rat [16] has shown even greater specificity and catalytic efficiency for retinal as a substrate. Immuno-histochemical analysis of mouse embryos shows that ALDH1A1 expression occurs primarily in cranial tissues, while ALDH1A2 expression occurs primarily in trunk tissue [17]. In addition, the targeted knockout of the *ALDH1A2* gene in mice results in a significant decrease in retinoic acid biosynthesis and early embryo death [18].

The development of inhibitors which can target individual ALDH enzymes provides one approach for addressing the role of a specific ALDH enzyme in the oxidation of retinal to retinoic acid in a given cell type. We have identified 4-(N, N-dipropylamino)benzaldehyde (DPAB) (Figure 1B) and 4-(N, N-diethylamino) benzaldehyde (DEAB) as potent, selective inhibitors of ALDH1A1, but not ALDH2 or ALDH3, with the dipropyl analog exhibiting 10-fold greater efficacy for inhibition [19,20]. The effect of DPAB on ALDH1A2 has not been reported.

DEAB was first used to sensitize the mouse leukemic cell line L1210/CPA, which is resistant to 4-hydroperoxycyclophosphamide (4-HC) by virtue of its overexpression of ALDH1A1. Treatment of L1210/CPA cells *in vitro* with 50 µM DEAB abolished the tumor cells' resistance to 4-HC [19]. DEAB also exhibited *in vivo* efficacy as a ALDH1A1 inhibitor, as demonstrated by the toxicity to the intestinal crypt cells in mice receiving co-injections of DEAB and cyclophosphamide [21]. More recently, DEAB has been used to demonstrate that overexpression of human ALDH1A1 in transfected cell lines is sufficient to cause cellular resistance to the oxazaphosphorines [22] and 4-HC [23]. DEAB has also been an effective reagent in fluorescent-activated cell sorting (FACS) techniques to isolate human hematopoietic stem cells based on the expression of ALDH1A1 activity [5,6]. In contrast to DEAB and DPAB, daidzin – a natural product isolated from *Pueraria lobata* (the Kudzu plant) – shows specificity for inhibiting ALDH2 at concentrations similar to that of DPAB used to inhibit ALDH1A1 [24,25]. An inhibitor specific for ALDH1A2 has not yet been reported.

In normal hematopoiesis, terminally differentiated cells are generated daily from a limited number of pluripotent stem cells. The stem cell population must be exquisitely regulated to ensure sufficient self-renewal as well as commitment to progenitor cells which can give rise to mature erythrocytes, platelets, lymphocytes, granulocytes, and macrophages. Retinoic acid appears to elicit a complex response of cell proliferation and/or commitment to a more differentiated cell type, depending on the differentiation state of the cell receiving the signal [26,27].

The HL-60 human promyelocytic leukemia cell line responds in culture to sub-micromolar concentrations of retinoic acid (*all-trans* and *13-cis*) by undergoing terminal differentiation to a granulocytic cell with the ability to phagocytose and reduce nitroblue tetrazolium [28]. The response to retinoic acid in HL-60 cells is dependent on the expression of the retinoic acid receptor RAR α [29]. The use of *all-trans* retinoic acid in differentiation therapy for patients with acute promyelocytic leukemia has resulted in prolonged, complete remissions when combined with cytotoxic chemotherapy, and may provide complete, long-term remissions as a single agent [30].

However, the enzyme or enzymes responsible for the formation of retinoic acid in the HL60 cells have not been identified. In one report, ALDH1A1 protein was not detected by Western blot, but enzyme activity was detected using flow cytometry and a fluorescent aldehyde substrate [5]. In a second study, ALDH1A1 protein was detected using an ELISA assay and activity was detected using aldophosphamide as substrate [31]. In these two studies, designed to look at the relationship of ALDH1A1 activity

to antitumor drug resistance, the levels of ALDH1A1 were low in HL-60 compared to other cell types, corresponding to the sensitivity of HL-60 cells to oxazaphosphorines. ALDH activity has been reported in HL-60 cells using 4-hydroxynonenal as substrate [32]. However, since this molecule is such a poor substrate with ALDH1 compared to ALDH3, the measured activity likely represents only ALDH3 activity. ALDH3 does not oxidize *all trans* retinal. Expression of ALDH1A2 activity in HL-60 cells has not been reported. Therefore, we chose the HL-60 cell line to assess the role of ALDH1A1 in the oxidation of retinal.

The collection of results demonstrating that retinal is an excellent substrate for ALDH1A1 *in vitro*, that ALDH1A1 is responsible for the synthesis of retinoic acid in the cranial tissues of the mouse embryo, and that hematopoietic stem cells are characterized by high expression of ALDH1A1, have led to the hypothesis that the oxidation of retinal to retinoic acid is a key physiologic role for ALDH1A1 in hematopoietic stem cells. Our long-term goal is to use DPAB to investigate the role of ALDH1A1 in retinoic acid biosynthesis at different stages of hematopoiesis. In this paper we show that DPAB is a potent inhibitor of retinal oxidation by murine and human ALDH1A1. However, DPAB only weakly inhibits the retinal-induced differentiation of HL-60 cells, suggesting that ALDH1A1 is not the operative retinal oxidizing enzyme in these cells.

Results and discussions

DPAB is a potent inhibitor of retinal oxidation with IC_{50} values of 0.11 and 0.13 µM for purified mouse and human ALDH1A1, respectively (Table 1). These IC_{50} values are 100-fold lower than the retinal concentrations used in the assay. The IC_{50} values for mouse ALDH1A1 are between the values observed with propanal (0.040 µM) and phenylacetaldehyde (0.36 µM) as substrates, with the human ALDH1A1 showing the same inhibition with retinal or propanal as substrate. The IC_{50} values reported were initially obtained from spectrophotometric assays, and repeated with an HPLC assay. We were concerned with using a spectrophotometric assay for these inhibition studies due to the presence of several absorbing species at 340 nm, including NADH, DPAB, retinal, and retinoic acid. Thus, we modified an HPLC assay in order to directly measure the amount of retinoic acid product formed. Previous reports of HPLC assays for ALDH reactions with tissue extracts used a single time point at 60 min to measure accumulation of product, after documenting that retinoic acid formation was still linear in time course experiments [33]. In our hands, the reaction with purified enzyme was only linear for product formation up to 10–15 minutes.

Following continuous exposure to 1 µM retinoic acid for 96 hours, almost 40% of cells have differentiated to gran-

Table 1: IC50 values for the inhibition by DPAB of the oxidation of selected aldehyde substrates by mouse and human ALDH1A1

Substrate	IC50 of DPAB (μM) ALDH1A1	
	Mouse	Human
all-trans Retinal	0.11 ± 0.02	0.13 ± 0.03
Propanal	0.040 ± 0.01	0.14 ± 0.04
Phenylacetaldehyde	0.36 ± 0.04	1.2 ± 0.1

IC50 values are the mean ± average deviation from at least 3 spectrophotometric assays, each performed in triplicate.

ulocytes, as seen by the positive NBT staining. Exposure to 1 μM *all-trans* retinal for 96 hours induced differentiation equivalent to exposure to 0.05–0.1 μM retinoic acid. With co-exposures to 1 μM retinal and up to 50 μM DEAB or DPAB, HL-60 differentiation was inhibited by only 30% after 144 hours of exposure, compared with cells exposed to retinal alone (Figure 2) DPAB did not alter the induction of differentiation by retinoic acid, nor did it affect cell viability up to 144 hours. Since DPAB's inhibition of cell differentiation in cells exposed to retinal was so low, we needed to demonstrate that chemical oxidation or any serum enzymatic oxidation of retinal to retinoic acid was not occuring under our culture conditions. Control dishes containing media and retinal were incubated for 144 hours, with aliquots removed every 24 hours and analyzed by HPLC. By day 6, less than 1% of the retinal was oxidized to retinoic acid, suggesting that non-enzymatic formation or serum-mediated formation of retinoic acid was not responsible for the observed differentiation in cells exposed to DEAB or DPAB.

Although DPAB is a more potent inhibitor of human ALDH1A1 than DEAB, we were concerned that DPAB was not remaining in solution at 50 μM in the culture media. However, the observation that the addition of DEAB, a much more soluble compound, resulted in the same percent inhibition, suggested that solubility was not a factor. Thus, artifacts due to solubility of inhibitor and extracellular oxidation of retinal do not appear to contribute to the observed response of DPAB on retinal oxidation.

In addition, the NAD-dependent oxidation of propanal or retinal was less than 0.05 nmoles NADH formed/min-10^7 cells in spectrophotometric assays using HL-60 cell extracts, even in the absence of DEAB or DPAB. The NAD-dependent oxidation of these aldehydes was not reproducibly detected above background in spectrofluorometric assays. Therefore, though low levels of ALDH1A1 activity have been reported in HL-60 cells in previous

studies [5,31], it appears that the differentiation observed in cells exposed to retinal alone or retinal plus DPAB was not mediated by ALDH1A1 oxidation of retinal to retinoic acid. One possibility is that a cytochrome P450 (CYP) enzyme is catalyzing the oxidation. Raner et al [34] and Chen et al [35] have described the formation of all-trans retinoic acid from retinal and retinol by members of the CYP1 and CYP3A families. In addition, expression of CYP1A1 and CYP3A4 mRNA has been reported in HL-60 cells [36]. The contribution of ALDH1A2 also can't be ruled out. However, its activity should be detected by the spectrophotometric or fluorometric assays.

Although ALDH1A2 appears only to catalyze the oxidation of *all-trans* retinal to *all-trans* retinoic acid, both ALDH1A1 and CYP1 and CYP3A [34] enzymes are capable of oxidizing *9-cis* retinal to *9-cis* retinoic acid. The family of retinoic acid receptors includes those that bind only *all-trans* retinoic acid (RAR α,β,γ and those that bind either *all-trans* retinoic acid or *9-cis* retinoic acid (RXR α,β,γ Since the transcriptional regulation of the retinoic acid ligand/receptor complex appears to involve the binding of receptor heterodimers (RAR/RXR), the specific cellular response may depend on the ratio of *all-trans* to *9-cis* retinoic acid formed, as well as the expression of receptor subtypes.

Our results suggest an intriguing possibility that regulation of retinoic acid formation in the HL-60 cells, and the subsequent terminal differentiation to granulocytes, could be mediated by one enzymatic acitivy, e.g. a CYP-mediated oxidation of retinal, whereas regulation of retinoic acid formation in the hematopoietic stem cells, and the subsequent maintenance of the self-renewal capacity of the hematopoietic stem cells, is mediated by ALDH1A1 oxidation of retinal (Figure 3). This hypothesis can be tested by using global or specific CYP inhibitors in the presence of retinal in HL-60 cells and by using DPAB in the presence of retinal in an hematopoietic stem cell assay system. We are attempting to assess the ability of DPAB to block retinoic acid formation in cultured, mouse hematopoietic stem cells.

Conclusions
We have shown that DPAB can serve as a potent inhibitor of purified human and mouse ALDH1A1 and should be a useful molecule to assess the role of ALDH1A1 in retinal oxidation in a cell system. In addition, our results indicate that in HL60 cells, the prototype cell line for studying retinoic acid induced cell differentiation, ALDH1A1 is not the operative oxidizing enzyme for retinal.
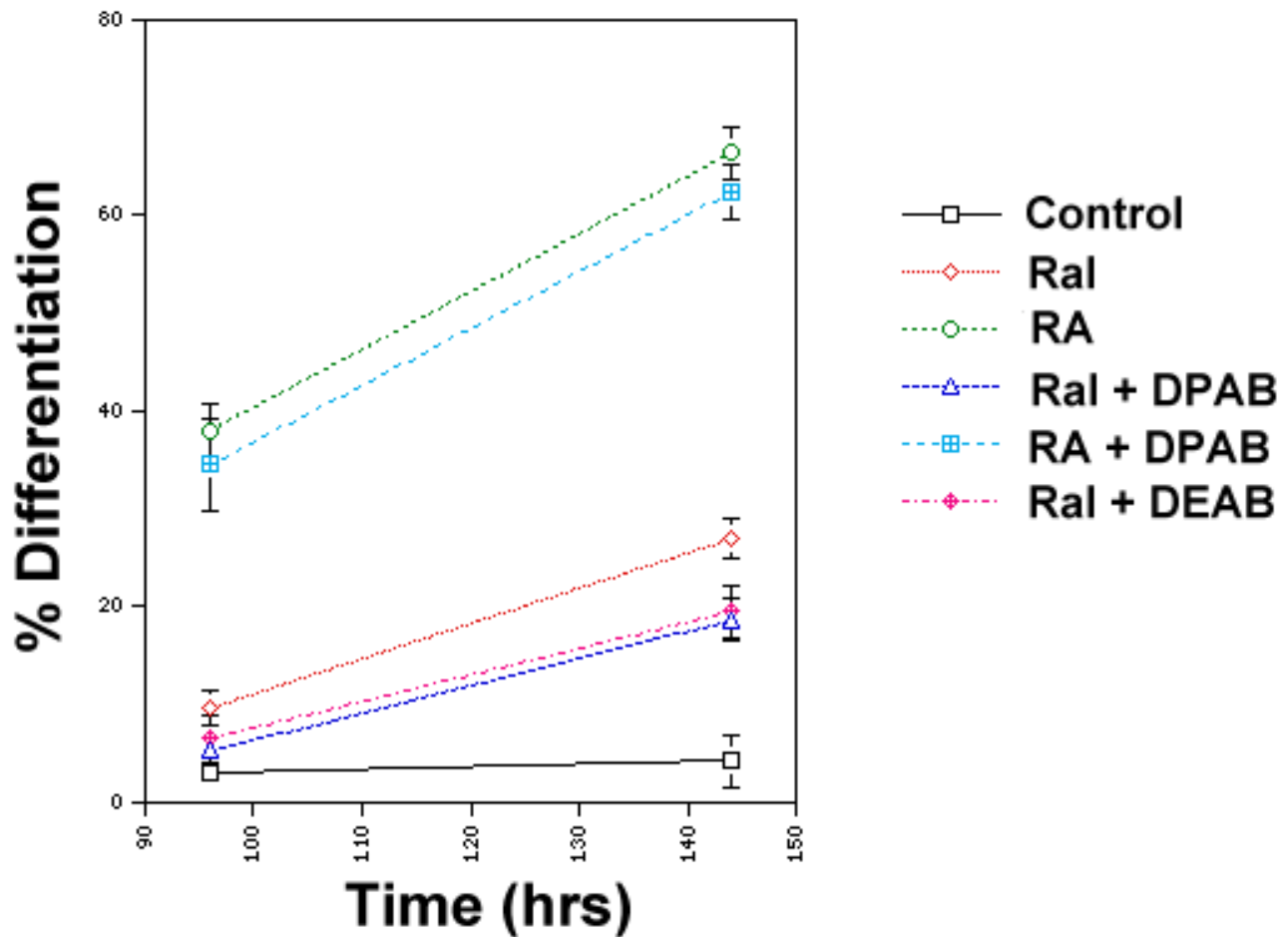
Figure 2
Differentiation response of HL-60 cells to retinoids and ALDH inhibitors. Retinoid concentrations were 1 μM for *all-trans* retinal (Ral) and *all-trans* retinoic acid (RA). Inhibitor concentrations were 50 μM for 4-(N,N-dipropylamino)benzaldehyde (DPAB) and 4-(N,N-diethylamino)benzaldehyde (DEAB). Control (black squares). Ral (red diamonds). RA (green circles). Ral + DPAB (dark blue triangles). RA + DPAB (hatched light blue squares). Ral + DEAB (hatched dark red diamonds).

Materials and methods
Materials
NAD, *all-trans* retinal, *all-trans* retinoic acid, dimethylsulfoxide, mammalian protease inhibitor cocktail, nitroblue tetrazolium, and pyrazole were obtained from Sigma Chemical Co., St. Louis, MO. Phenylacetaldehyde, 4-(N,N-diethylamino) benzaldehyde, and propanal were purchased from Aldrich Chemical Co., Milwaukee, WI.

Purification of ALDH1A1 from mouse liver and human erythrocytes
The purification of ALDH1A1 from mouse liver and human erythrocytes was performed as in [20].

Substrate and inhibitor preparations
Retinal and retinoic acid were dissolved in either DMSO or ethanol and stored as 100 mM stock solutions in foil-wrapped vials at -20°C. For enzyme assays, retinal prepared in DMSO was diluted in a 70% methanol: 30% water (v/v) solution and all assays were run under dim light. DPAB was synthesized as described in [20] and stored as a yellow oil at 4°C. DPAB was diluted in DMSO to a stock concentration of 10 mM and then diluted in water prior to enzyme assays. DMSO concentration did not exceed 0.1% (v/v) in the assay and this concentration had no effect on enzyme activity.
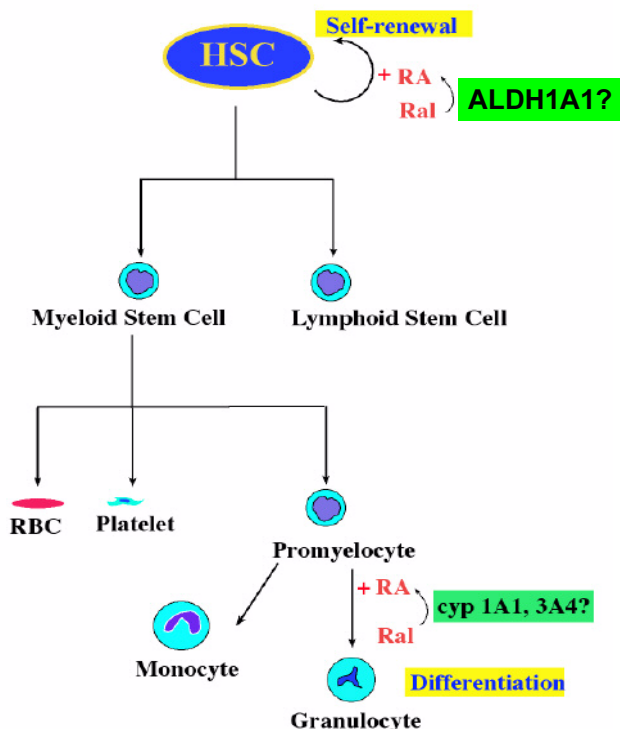
Figure 3
A schematic depicting the potential roles of different oxidative enzymes in retinoic acid biosynthesis and the differentiation vs. self-renewal response to the retinoic acid ligand at different stages of hematopoiesis. Hematopoietic stem cell (HSC). *All-trans* retinal (Ral). *All-trans* retinoic acid (RA).

Spectrophotometric enzyme assays

ALDH1A1 activity was measured on a Shimadzu UV1201 Spectrophotometer at 36.5° (± 0.5°) at 340 nm. Standard assay conditions were as follows: 1 mL final reaction volume; assay buffer (0.1 M sodium pyrophosphate pH 8.4, 1.0 mM EDTA, 5.0 mM dithiothreitol); 1.0 mM NAD; 10 mM pyrazole. The order of addition in the assay was as follows: buffer; pyrazole; NAD; ALDH (10 mU mouse liver ALDH1A1; 5 mU human erythrocyte ALDH1A1; or HL-60 cell extract); inhibitor (if any); and lastly, the aldehyde substrate. One Unit of activity is defined as 1 μmole NADH formed /minute. Background activity was determined for 60–90 seconds after the addition of enzyme and the reaction rate was measured during the 30–60 seconds following addition of retinal substrate Propanal concentration was varied from 500 to 10 μM (20–0.4 times K_m), phenylacetaldehyde concentration was varied from 40 to 5 μM (5–0.6 times K_m), and retinal concentration was varied from 1–10 μM, all with constant (1 mM) NAD

concentration. DPAB concentrations were varied from 20 μM to 0.05 μM. All trials were done in triplicate.

HPLC enzyme assays

Since there are three molecules with strong absorbances at 340 nm (retinal, retinoic acid, and NADH), we developed a high pressure liquid chromatography (HPLC) assay to assess the ability of DPAB to inhibit retinal oxidation by class 1 ALDH. Assay conditions were: 0.5 mL final reaction volume; assay buffer (0.1 M sodium pyrophosphate, pH 8.4, 1.0 mM EDTA, 5.0 mM dithiothreitol or glutathione); 1.0 mM NAD. The reaction rate was measured at 37°C for 1–10 minutes. The reaction was stopped by the addition of 200 μL butanol. After vortexing for 1 minute, 50 μL of saturated K_2HPO_4 was added. The sample was vortexed and centrifuged for 2 minutes at 16,000 g. 50 μL of the resulting organic layer was injected onto a reverse phase C18 column (Phenomenex). The mobile phase was 0.5% acetic acid: 20% water: 79.5% acetonitrile (v/v) with a flow rate of 2.0 mL/min. *All-trans* retinal and *all-trans* retinoic acid were detected at 360 nm with retention times of 10.0 and 7.7 minutes, respectively.

HPLC assay for detection of retinal purity and stability in culture

HPLC was used to analyze the purity of our retinal samples and the stability of retinal under culture conditions. The stability of retinal in tissue culture was determined by incubation of 10 μM retinal in culture media (RPMI 1640 plus 15% fetal bovine serum) for six days at 37°C. At 24 hour intervals, 1 mL of media was removed. 200 mL of 50% butanol:50% acetonitrile (v/v) was added and the mixture was vortexed for 60 seconds, followed by centrifugation for 2 minutes at $16,000 \times g$. The top organic layer was removed and diluted 1:5 in acetonitrile before injection onto the HPLC column.

Cell differentiation assays

HL-60 cells (ATCC, Rockville, MD) were propagated in RPMI-1640 media supplemented with 15% fetal bovine serum and 50 U/mL penicillin and 0.5 mg/mL streptomycin. Cells were subcultured and treated at 2×10^5 cells/mL. Cells were exposed to combinations of 1 μM retinoic acid, 1 μM retinal, and various concentrations of DPAB and DEAB prepared in sterile dimethyl sulfoxide (DMSO) or ethanol. Final concentrations of DMSO or ethanol in the culture medium did not exceed 1% v/v. Cells were exposed for up to 144 hours without replacement of media or washing out of retinoid or inhibitor. Cell differentiation was measured by mixing 100 μL aliquots of cells with 100 μL of a solution containing 1.0% w/v bovine serum albumin, 0.2% w/v nitroblue tetrazolium and 1.0 μg/mL of 12-O-tetradecanoylphorbol 13-acetate (TPA). The mixture was incubated for 45 minutes at 37°C in a 24-well plate. Cells were pipetted onto a Bright Line counting

chamber and scored as positive for differentiation by the presence of purple NBT precipitate using bright field microscopy. Two observers each counted two fields of at least 200 cells from each well of treated cells. Cell survival was determined by Trypan blue dye exclusion.

Spectrofluorometric assays for ALDH activity in HL-60 Cells

Cytosolic extracts of HL-60 cells were analyzed for ALDH activity using a Perkin Elmer 650 spectrofluorometer with an excitation wavelength of 350 nM and an emission wavelength of 460 nM. Assay conditions were as given for the spectrophotometric enzyme assays above, with propanal or *all-trans* retinal used as the aldehyde substrate.

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